Chapter Two

Detection Methods and Alternate Indicator Organisms [1]

2.1 Introduction

Public demand and regulatory requirements compel monitoring for pathogen risks. Such monitoring requires feasible and accurate detection methods for appropriately selected microbes. Water quality monitoring in the U.S. is most frequently conducted for bacterial indicators using the standard membrane filtration or multiple tube fermentation/most probable number methods. U.S. EPA requires that a Total Maximum Daily Load (TMDL) be developed for water bodies violating standards, which are determined using the monitoring results. The TMDL is generally developed for the microorganism responsible for the violation. There are exceptions, however, such as when there are waterborne disease outbreaks. In these instances, other detection methods may need to be employed to identify causative agents and determine their presence and concentrations in a watershed.

Microorganisms responsible for waterborne disease outbreaks are identified through clinical testing of individuals who seek medical care for their illness. Illnesses are classified as waterborne disease outbreaks when more than one individual is found to be infected with the same microbe believed to be from a common source of drinking or recreational water. Environmental officials assigned to investigate and manage the pollution responsible may use microbial source tracking and pathogen detection methods to investigate possible sources and determine the extent of contamination.

This chapter presents information on detection methods for bacteria, viruses, and protozoa, summarized in Tables 2-1, 2-2, and 2-3, respectively. In the section on bacteria, detection methods for both indicators and pathogens are discussed, as well as alternatives to the traditional indicator organisms and an overview of selected methods for microbial source tracking. Although helminths and fungi are discussed in Chapter 1, their methods were not reviewed for this chapter due to the high unlikelihood that these organisms will be encountered in urban watersheds in the U.S. Information about pathogenic fungi is available in *Standard Methods for the Examination of Water and Wastewater* (Clesceri *et al.*, 1998), hereafter referred to as *Standard Methods*. A method for helminth ova is presented in the U.S. EPA document *Control of Pathogens and Vector Attraction in Sewage Sludge* (U.S. EPA, 1999) available at http://www.epa.gov/ORD/NRMRL/Pubs/1999/625R92013.pdf.

[1] *Much of this chapter is excerpted from Monitoring Pathogens in the Watershed: Indicator Organisms and Detection Methods, submitted for peer-reviewed publication by M.E. Tuccillo and J.M. Perdek, and Investigating Watershed Microbial Pathogen Contamination to Manage Public Health Risks, submitted for peer-reviewed publication by J.M. Perdek, M.E. Tuccillo, and S.M. Wankel*.

2.2 Detection Methods

2.2.1 Bacteria

2.2.1.1 Cultural and Enzyme-Based Methods

Cultural methods, or those that grow bacteria in a prepared medium, have been used for indicator bacteria detection and enumeration for over a century (Pyle *et al.*, 1995). Membrane filtration methods are well established and routinely used. The details of these methods are described in *Standard Methods*. The water sample is filtered, the filters are incubated on a growth medium for a specific time and temperature, and the resulting colonies are enumerated. The membrane filtration incubation period is 24 hours for fecal coliforms and total coliforms, but for other bacteria can take longer; *Staphylococcus aureus* and *enterococcus* cultures must be incubated for 48 hours, and *Pseudomonas* cultures should be incubated for 72 hours. An improved U.S. Environmental Protection Agency (EPA) method for *enterococcus* using a modified type of agar (mEI) requires only 24 hours of incubation (U.S. EPA, 1997). The membrane filtration methods require confirmation tests, which entail further effort and additional incubation time.

Multiple-tube fermentation/most probable number methods for coliform bacteria are based on the ability of the organisms to ferment lactose. Tubes with growth medium are inoculated with a series of undiluted and diluted samples, with several tubes inoculated per dilution. Following incubation at the specified temperatures, the numbers of tubes demonstrating a positive response are recorded and a statistical estimate of the bacterial density is determined. Most probable number methods take 48 hours for coliform incubation, plus an additional 24-hour confirmation test. *Enterococcus* and fecal streptococcus are incubated for 24-48 hours with an additional 24 hours for confirmation (Clesceri *et al*., 1998). Fecal coliforms, however, can be analyzed in 24 hours by the A-1 broth 1-step method (*Standard Methods* # 9221E.2).

Methods that rely on counting the colonies that form during incubation, including membrane filtration, tend to underestimate bacterial numbers (Sartory *et al*., 1999). This phenomenon affects analyses for both indicators and pathogens. This may be due to clumping, particle association, cell injury, and the viable-but-nonculturable (VBNC) state of the bacteria. In the VBNC state, cells may maintain viability and metabolic activity, but fail to grow and multiply on culture plates. Huq and Colwell (1996) reviewed this topic with special attention to *Vibrio cholerae*, although this condition applies to *Aeromonas*, *Shigella*, *Staphylococcus*, and *Campylobacter*, among others. Such underestimation of bacterial counts presents the obvious danger of giving rise to misleading reports.

Substrate hydrolysis by a specific enzyme with colorimetric endpoints forms the basis of several detection methods. In substrate hydrolysis, the hydrolysis reaction between an enzyme in the bacteria and the substrate results in a color change that is used to determine the analytical results. Cultural methods for *E. coli* are based on detecting the action of the enzyme β-

glucuronidase upon the substrate 4-methylumbelliferyl-β-D-glucuronide (MUG) (Sartory *et al*., 1999; Shadix *et al*., 1991). The product fluoresces blue under long wavelength ultraviolet (UV) light, indicating the presence of *E. coli*. The *E. coli* technique in *Standard Methods* requires additional incubation of coliform-positive membrane filtration samples to test for MUG utilization by β-glucuronidase. The U.S. EPA method using membrane-Thermotolerant *E. coli* (mTEC) agar for *E. coli* analysis (U.S. EPA, 1985) relies upon detection of the enzyme urease; a modified mTEC method relies upon β-glucuronidase. Substrate hydrolysis by β-galactosidase is used for detection of thermotolerant coliforms. The rapid method tested by Robertson *et al.* (1998) uses only a 6-hour incubation to test for β-glucuronidase for *E. coli* and β-galactosidase for thermotolerant coliforms.

There are rapid alternatives to membrane filtration methods based on enzyme substrate utilization by coliform bacteria and *enterococci*. IDEXX Laboratories (Westbrook, ME) produces a series of widely used EPA-approved products. Their Colilert® Quantitray™ , which uses their patented Defined Substrate Technology, is an easy-to-use commercial most probable number method designed for simultaneously determining the presence of total coliforms and *E. coli* in 24 hours (Edberg *et al.,* 1989; Townsend *et al*., 1996). Total coliforms are detected by the action of β-galactosidase, and *E. coli* detection is based on the action of β-glucuronidase. Coliforms produce a yellow product and *E. coli* produces a product that fluoresces yellow. Colilert-18® permits detection of these organisms in only 18 hours. Colilert® has been shown by some researchers to be as sensitive as Multiple Tube Fermentation (MTF) and membrane filtration (Eckner, 1998; Fricker *et al*., 1997; Edberg *et al*., 1990). Francy and Darner (2000) used recreational water to compare Colilert to the U.S. EPA-recommended mTEC method (U.S. EPA, 1985), a β-glucuronidase-based membrane filtration technique. The authors found statistically significant differences between the methods, but note that their test area was small and further work is needed. The expression of β-glucuronidase can, however, be suppressed by environmental stress (Sartory *et al*., 1999; Edberg *et al*., 1990), raising the possibility of underestimating bacterial densities. Furthermore, *E. coli* O157:H7 does not possess this enzyme, so a separate test for *E. coli* O157:H7 would be needed if it is suspected.

Similar to Colilert®, IDEXX Laboratories' enzyme-based Enterolert® method is designed to provide a most probable number method in 24 hours for *enterococcus* in water. The hydrolyzation product of the substrate fluoresces blue. Abbott *et al.* (1998) found a positive correlation between Enterolert® and membrane filtration in marine waters in New Zealand. Budnick *et al.* (1996) and Eckner (1998) reported equal or better sensitivity and specificity with Enterolert compared to membrane filtration in recreational waters.

Because indicator bacteria are used as a basis for public health decisions in dynamic aquatic environments such as beaches, long analysis times are problematic because levels of *E. coli* and thermotolerant coliforms fluctuate. Fortunately, there are rapid method alternatives to the commonly used cultural methods can speed decision-making about protective measures such as beach closings. The 18-hour incubation time for Colilert and the 6-hour incubation used in the method of Robertson *et al.* (1998) are two examples of incubation methods that require less time. In addition to rapid cultural methods, other classes of detection methods, such as immunological and genetic techniques, offer possibilities for faster analysis times.

2.2.1.2 Immunological Methods

A group of immunological detection methods for microorganisms is based on the use of antibodies, which bind with antigens on the organism's surface. A limiting factor with all immunological techniques is the specificity of the antibody used. Ideally an antibody should bind only with a single antigen, thereby targeting only the organism of concern. Monoclonal antibodies (Mabs) are clonally derived from a single antibody-producing cell. This means that they are exceptionally pure and highly specific in their action.

Some immunological methods are applicable for efficient bacterial detection methods. In immunofluorescence (IF), the antibodies are tagged with a dye that fluoresces under UV light; enumeration can be accomplished by epifluorescent microscopy. Sartory and Watkins (1999) note that there is promise for a limited cultural period (4-6 hours) coupled with detection either by substrate light emission or immunological techniques for same-day results. In their review of rapid methods, McFeters *et al.* (1999) cite examples of the staining of bacteria with fluorescent antibodies performed directly on membrane filters. This avoids steps such as sample concentration and fixation on glass slides.

Because pathogenic *E. coli* O157:H7 does not produce β-glucuronidase, the *E. coli* procedures in *Standard Methods* will not detect it without additional steps. Immunological techniques may be useful in situations where this pathogen is suspected. The rapid *E. coli* O157: H7 methods of Pyle *et al.* (1995, 1999) involve incubation with a dye that indicates viability, followed by fluorescent antibody staining and enumeration by epifluorescent microscopy or laser scanning cytometry (the study and measurement of cells). Kfir *et al.* (1993), however, caution against problems of specificity with the use of monoclonal antibodies as a rapid tool for detecting fecal bacteria in water, and in particular *E. coli*.

Commercially available instruments such as Chem Scan® can detect and enumerate fluorescent bacteria (McFeters *et al.*, 1999), further facilitating rapid detection methods. Commercial sensors continue to be developed and were reviewed by Ivnitski *et al.* (1999). Some are immunologically based; others rely on enzyme detection or nucleic acid detection. A rapid immunological technique for *E. coli* O157 and *Salmonella typhimurium* (Yu and Bruno, 1996) uses a commercial sensor and shows promise as a screening tool, identifying samples that should be further analyzed. These simplified, commercial screening tools provide additional options for situations where easy, rapid screening is desired.

A process called enzyme-linked-immunoabsorbent assay (ELISA) tags an antibody with an enzyme. After incubation, an enzyme substrate is added, and the formation of a pigmented product is indicative of the amount of enzyme present in the sample and, therefore, the amount of microorganism in the sample (Bitton, 1980). Advantages of ELISA are that it is robust, versatile and simple to perform (Kfir *et al.*, 1993). As with any immunoassay, limitations are related to the specificity of the antibody used. Various easy-to-use commercial ELISA kits are available, such as the Wellcolex kits (Murex Biotech Dartford, United Kingdom). Developed mostly for clinical or food applications, these techniques may be useful for water quality testing when simple techniques are desired. Limited trials with wastewater have, however, raised the

possibility of cross reactions with competing organisms in the samples (Meckes, 2001). Further testing of these kits with environmental waters is needed.

2.2.1.3 Genetic Methods (Gene Probes and PCR)

Development of genetic methods has provided new sensitive options for pathogen detection. Gene probes are nucleotide sequences that pair with corresponding sequences in the sample through a process called hybridization (Hurst *et al*., 1989). The probes make good detection tools and can be labeled with a radioisotope, an enzyme, or a fluorescent chromogene to permit detection. Although genetic methods require more sophisticated equipment and techniques than cultural methods, there are commercially available gene probe kits that only require typical microbiological laboratory equipment and are easy to use. For example, Gene-Trak (Hopkinton, MA) produces gene probe assays for several organisms including *E. coli* and *Salmonella.* The kits, which are geared primarily toward food or clinical applications, have a colorimetric endpoint and come with a photometer. Rice *et al.* (1995) found that the probe performed well with pure cultures, but failed to detect seven of thirteen positive cultures in creek and river samples, possibly due to low bacterial densities in the natural waters. The authors note that further research is needed to improve the performance of the method with environmental samples, possibly through increased enrichment or larger sample aliquots.

The polymerase chain reaction (PCR) has greatly improved the ability to detect low densities of pathogens in environmental samples. PCR produces many copies of a target section of a microorganism's deoxyribonucleic acid (DNA). With the large number of copies produced by PCR, the target DNA can be detected using gene probes or gel electrophoresis (Toze, 1999). Gel electrophoresis is a process used to impart an electric current to DNA fragments in a gel of specific density. Different size fragments move at different rates and can be visualized as a series of bands in the gel. The use of PCR offers several advantages, including specificity, sensitivity, rapidity, accuracy, and the capacity to detect small amounts of target nucleic acid in a sample. PCR-based methods can be used both to rapidly identify bacteria that have been isolated and for direct pathogen detection in environmental samples (Toze, 1999).

Several researchers have published protocols for PCR-based detection of *E. coli* in water (Fricker *et al*., 1999; Kong *et al*., 1999b; Tsen *et al*., 1998). The method of Fricker *et al.* (1999) is especially quick, identifying *E. coli* from membrane filters within two hours. Tsen *et al.* (1998) use an 8-hour pre-culture step, and claim detection of 1 cfu per 100 mL. By combining PCR and radiolabeled gene probes, Bej *et al.* (1990) developed a sensitive and specific method for *E. coli*, *Salmonella* and *Shigella spp.* A PCR method for *Salmonella spp.* published by Way *et al.* (1993) can also detect other coliform bacteria (e.g., *Shigella*, *E. coli* and *Citrobacter*), rendering the technique very useful for environmental samples. Palmer *et al.* (1993) found PCR to be sensitive and specific for *Legionella* in sewage treatment plant influent and in ocean receiving waters. A method for detecting *Aeromonas* in seawater (Kong *et al.*, 1999a) may be useful for monitoring because of the prevalence of *Aeromonas spp*. in the aquatic environment.

There are, nevertheless, several disadvantages to PCR-based methods. They require specialized equipment and skilled technicians (Toze, 1999). The results of PCR alone do not

provide a means for quantification; they indicate presence or absence of the target genetic material. Furthermore, PCR alone does not directly provide information about the viability or infectiousness of the organisms because DNA may persist in the environment (Alvarez *et al*., 1993; Gantzer *et al*., 1999; Kopecka *et al*., 1993; Metcalf *et al*., 1995; Sobsey *et al*., 1998a). These techniques are still at the research stage and are beyond the capabilities of most state and local municipalities for routine analyses. However, they may eventually become a viable option for routine pathogen analysis and may be especially useful for studies characterizing the identities and sources of pathogens within a watershed.

2.2.2 Viruses

Current routine monitoring strategies do not test for viruses; they rely on indicator bacteria. Various viruses (e.g., rotavirus, adenovirus, hepatitis A virus and Norwalk-like viruses) are important agents of illness in sewage-polluted waters (Metcalf *et al.*, 1995). There are clearly cases where virus identification is needed, such as in investigations of outbreaks or in research studies. In cases where direct detection of viruses is needed, a variety of methods exists and new methods continue to be developed.

2.2.2.1 Sample Concentration

Because of the low concentrations of viruses in environmental samples, methods used to detect enteric viruses require an initial concentration step to make them detectable. For environmental waters this is typically accomplished by sorption of viruses onto a filter. According to Schwab *et al.* (1993), hundreds to thousands of liters of water may need to be filtered through a special filter cartridge to achieve sufficient virus concentration for detection. A yarn fiber filtration cartridge or a cartridge with pleated sheets of filter material are particularly useful because of field portability. After filtration, the viruses are generally recovered from the filter into about 1L of eluant. *Standard Methods* describes techniques for virus concentration by adsorption to and elution from microporous filters. Beef extract is one of the most common eluants (DeLeon and Sobsey, 1991; Schwab *et al.*, 1993). A secondary concentration step may be needed, such as ultrafiltration or flocculation. In their review of filtration and elution methods, DeLeon and Sobsey (1991) caution that humic and fulvic substances in water may interfere with virus sorption onto filters. They also point out that adsorption/elution efficiencies vary for different viruses; for some the recoveries are low.

Table 2-1. Summary of Detection Methods for Bacteria			
Cultural and Enzyme-Based			
Method	**Duration**	**Results Provided**	**Capabilities Needed**
Membrane Filtration	24 hours or longer depending on bacteria + 24-hour confirmation	Enumeration, Presence-Absence	General Microbiology Laboratory
Multiple Tube Fermentation/Most Probable Number (MTF/MPN)	24 hours or longer depending on bacteria + 24-hour confirmation	Enumeration, Presence-Absence	General Microbiology Laboratory
Substrate Hydrolysis – Colorimetric	6 to > 24 hours depending on method and organism	Presence-Absence	General Microbiology Laboratory
Defined Substrate Technology	*E. coli* and Total Coliform – 24 hours; *Enterococcus* – 24 hours	Enumeration	General Microbiology Laboratory
Immunological			
Immunofluorescence (IF)	< 24 hours	Enumeration by epifluorescent microscopy	Specialized Microbiology Lab.
Commercially Available Instruments	< 24 hours	Enumeration	General Microbiology Laboratory
Enzyme-Linked-Immunoabsorbent Assay (ELISA)	Varies	Enumeration	Kits available for clinical and food applications; more research for environmental app. needed
Genetic			
Gene Probes	Time varies	Presence-Absence, Enumeration in research stage	Kits available for clinical and food applications; more research for environmental app. needed
PCR	< 24 hours	Presence-Absence, Enumeration in research stage	Specialized Microbiology Lab.; techniques still in research stage

Table 2-2. Summary of Detection Methods for Viruses			
Method	**Duration**	**Results Provided**	**Capabilities Needed**
Cultural			
Cultural Assay	Varies, on the order of days	Presence-Absence, enumeration; indicates viability	General Microbiology Laboratory
Immunological			
Immunological	Varies	Enumeration by epifluorescent microscopy; Does not indicate viability	Specialized Microbiology Lab.
Immunological: ELISA	Varies	Presence-Absence; Enumeration	More research needed for environmental app.
Genetic			
Gene Probes	Varies	Presence-Absence by radioisotope or enzyme	Specialized Microbiology Lab.; More research for environmental app. needed
PCR	< 24 hours	Presence-Absence	Specialized Microbiology Lab.; techniques still in research stage

2.2.2.2 Cultural Assay

Several assay techniques are available for virus detection in the concentrated sample. Detection methods given in *Standard Methods* rely on the infection and destruction of host cells by the virus (cytopathic effects). In the plaque assay method, for example, a viral suspension is placed on a monolayer of cells, and areas of cell destruction due to infection (plaques) are enumerated and expressed as plaque-forming units (PFU). An advantage of cell culture is that it indicates viability. There are, however, disadvantages. Cell culture assays such as the plaque assay method require different cell lines for detection of different viruses. Although most enteric viruses can be cultured, some viruses, such as Norwalk virus, hepatitis A and E, calciviruses, rotaviruses and astroviruses either do not grow or grow slowly in cell culture assays (DeLeon and Sobsey, 1991; Metcalf *et al*., 1995). Thus, cell cultures cannot be used to detect several important pathogenic viruses.

2.2.2.3 Immunological Techniques

Immunological techniques are useful in virus detection. The viruses may be in suspensions, trapped on filters, or in cell cultures (Hurst *et al*., 1989). When they are trapped on filters, without some form of cell culture, the assay cannot indicate infectivity. As with the bacterial techniques mentioned earlier, use of a fluorescently-tagged antibody permits enumeration by epifluorescent microscopy. Oragui *et al.* (1989) have used immunofluorescence for detection of rotaviruses in wastewaters. Similarly, radioimmunoassay uses an antibody tagged with a radioactive isotope to bind to the viral antigen, and detection is accomplished by measuring the radioactivity of the antibody-antigen complex.

Variants of the enzyme-linked assay can detect viral antigens trapped on a filter or associated with infected cells (for viruses that can be cultured). ELISA has been used to detect Hepatitis A virus in tap water (Schnattinger, 1985). Nasser and Metcalf (1987) and Nasser *et al.* (1993) developed an amplified ELISA (A-ELISA) method for virus detection that has greater sensitivity than ordinary ELISA, as well as good specificity, speed, and low cost. Nasser *et al.* (1994) used A-ELISA to indicate the presence of viable poliovirus in water. According to Kfir and Genthe (1995), commercial clinical ELISA kits have been used for environmental waters and are available for some viruses, including rotaviruses and adenoviruses.

2.2.2.4 Gene Probes

Viruses may be detected by the use of gene probes. As with the immunological methods, the target material may be present in a solution, trapped on a filter, or present in infected cells. Detection may be accomplished via a radioisotope or enzyme attached to the gene probe. An effective method must specify a target nucleic acid sequence that is specific to the organism of concern. As with other assays, prior amplification by cell culture indicates that the viruses are infective. Hurst *et al.* (1989) note that hybridization is more sensitive and faster than plaque assays or immunofluorescence. According to Gerba *et al.* (1989), hybridization is much more sensitive than ELISA methods, and gene probes have been developed for the major groups of enteric viruses. Gene probes have been used for the detection of hepatitis A virus and other enteroviruses in drinking water samples that were negative by radioimmunoassay and that required weeks of propagation in cell cultures to be detectable by immunoassays (Shieh *et al*., 1991). Other examples of studies using gene probes include the detection of rotavirus in fresh and estuarine waters (Nasser *et al.*, 1991), enteric viruses in raw and treated waters (Genthe *et al.*, 1995), and poliovirus in sewage-contaminated groundwater (Margolin *et al.*, 1990). Margolin *et al.* (1993) found excellent agreement between cell culture and gene probe methods for a variety of environmental water samples. As noted earlier, however, genetic techniques require sophisticated equipment and techniques. The research studies show promise for efficient viral detection, but easy-to-use kits are not readily available.

2.2.2.5 PCR-based Methods

The polymerase chain reaction is particularly useful for virus detection because it amplifies the low quantities of viral genetic material present in environmental samples. The use

of PCR for detecting viruses offers many advantages over the traditional methods, including lower detection limits, increased range of viruses detectable, specificity, and shorter processing time (Toze, 1999). As with other methods, water samples may need to be filtered or otherwise concentrated first. Reverse transcriptase, a compound that catalyzes the formulation of DNA using RNA as a template (RT-PCR), is used when a virus' genetic material is RNA. The RT-PCR methods can detect less than 10 PFU of a virus in a filter eluate sample in less than two days.

Standard sample concentration procedures can pose problems for PCR. Humic acids, which cause interference, can be concentrated along with the viruses. Proteins and salts in beef extract eluant can also interfere with molecular methods (Schwab *et al*., 1993). It is, therefore, necessary to separate the viruses and their DNA from such impurities (Kopecka *et al*., 1993). The inhibitory problems in some samples have been avoided by using immunologic-based methods to capture viruses for subsequent PCR amplification (Metcalf *et al*., 1995; Schwab *et al*., 1996; Toze, 1999).

Polymerase chain reaction-based techniques have been used successfully for detection of viruses in various types of environmental samples, often with relatively short analysis times. Methods have been developed for astroviruses (Marx *et al.*, 1998), enteroviruses (Gilgen *et al.*, 1995; Griffin *et al.*, 1999; Vantarakis and Papapetropoulou; 1998, 1999), rotaviruses (Soule *et al*., 2000), and adenoviruses (Vantarakis and Papapetropoulou, 1998, 1999) in a variety of environmental waters. In a comparison of three detection methods for enteroviruses in activated sludge and sewage waters, Kopecka *et al.* (1993) found PCR to be vastly more sensitive than cell culture methods and direct hybridization. A number of RT-PCR methods offering various advantages have been devised. These include a triple RT-PCR method for the simultaneous detection of hepatitis A virus, poliovirus, and rotavirus (Tsai *et al*., 1994), an assay for enteroviruses with a tissue culture state to indicate infectivity (Fricker *et al*., 1999), and a relatively rapid method using RT-PCR, followed by hybridization and a form of ELISA (Greening *et al*., 1999).

2.2.3 Cryptosporidium and Giardia

2.2.3.1 Immunofluorescence

As with viruses, identification of *Cryptosporidium parvum* oocysts in water is not routine, limiting our ability to assess the public health threat from *Cryptosporidium* (Rose, 1997). The public health impacts of this organism are discussed in detail in Chapter 1. The detection procedure for *Cryptosporidium parvum* oocysts and *Giardia lamblia* cysts described in *Standard Methods* is an immunofluorescence (IF) procedure. To prepare the sample, hundreds of liters of water are passed through a filter cartridge. Cysts and oocysts are recovered from the cartridge, concentrated, and filtered onto a membrane. In addition to the epifluorescent microscopy phase, contrast microscopy is used for confirmation of the internal structures of the organisms. The newest U.S. EPA-recognized IF method for *Cryptosporidium* and *Giardia* (U.S. EPA, 2001) is a more streamlined method that entails filtration of only 10 L of water, uses well

slides instead of membrane filters, and uses differential interference contrast (DIC) microscopy for confirmation.

The IF procedures have low recoveries, are costly and time-consuming, and cannot indicate viability (Slifko *et al*., 1997). The most recent edition of *Standard Methods* acknowledges these limitations, but does not provide an updated method, noting that methods research is evolving rapidly. Allen *et al.* (2000) note that IF techniques have a high rate of both false positives and false negatives, rendering monitoring results highly suspect.

Two methodologies address the problem of viability. Jarmey-Swan *et al.* (2000) improved upon IF for *Giardia* cysts by staining with fluorescein diacetate prior to antibody staining. The combination of the two stains allows identification of viable cysts via microscope. Slifko *et al.* (1997, 1999) have developed and statistically standardized a detection method based on cell culture technology combined with an IF assay. The technique, called the Foci Detection Method (FDM), can be used to detect concentrations as low as 10 oocysts per sample. This method has good promise of being a specific test for *Cryptosporidium parvum*, but it has not yet been tested with all *Cryptosporidium* species.

Table 2-3. Summary of Detection Methods for *Cryptosporidium* and *Giardia*

Method	Duration	Results Provided	Capabilities Needed
Immunological			
Immunofluorescence	72-96 hours	Enumeration by epifluorescent and contrast microscopy; Does not indicate viability	Specialized Microbiology Lab.
Genetic			
Gene Probes	Time varies	Presence-Absence	Specialized Microbiology Lab.; more research for environmental app. needed
PCR	< 24 hours	Presence-Absence; does not indicate viability	Specialized Microbiology Lab.; techniques still in research stage

2.2.3.2 Gene Probes and PCR-Based Methods

While immunofluorescence remains the primary approach for *Giardia* and *Cryptosporidium* analyses, work is continually underway to devise improved techniques that may replace the current methods. Rose (1997) notes that PCR, ELISA, cultural, immunomagnetic separation (IMS), and colorimetric methods are not yet sufficiently developed for routine use. Below is an overview of methods employed in research studies; these may point the way for future routine detection options.

As an alternative to the antibody approaches, gene probes have been used with fluorescent staining of *Cryptosporidium parvum* oocysts in water (Vesey *et al*., 1998). Prescott *et al.* (1999) describe the use of gene probes for the detection of *Cryptosporidium parvum*. The method has good specificity and determines viability.

Studies using PCR for detection of *Cryptosporidium* and *Giardia* (Rochelle *et al*., 1997; Stinear *et al*., 1996; Ware *et al*., 1995) have shown that PCR has excellent sensitivity. Furthermore, simultaneous detection of *Cryptosporidium* and *Giardia* is possible. Wiedenmann *et al.* (1998) provide a thorough review of PCR for the detection of *Cryptosporidium parvum*. As with viruses, methods are available for separation of cysts and oocysts from substances that can inhibit PCR. For example, a technique called the Xtra Bind Capture System has been used to facilitate the concentration of *Cryptosporidium* from water prior to RT-PCR (Kozwich *et al.*, 2000). In this method, potential inhibiting contaminants were removed and PCR amplification was performed without needing to elute the oocysts from the capture material. The authors completed the analysis within only three hours. Other rapid and sensitive PCR methods combine immunomagnetic (magnetic beads with antibodies) separation of *Cryptosporidium* oocysts, followed by PCR for amplification and hybridization for detection (Hallier-Soulier and Guillot, 1999; U.S. EPA 2001). Champliaud *et al.* (1998), however, note difficulties differentiating between *Cryptosporidium parvum* and other nonpathogenic *Cryptosporidium* species using PCR. Furthermore, as with viruses, PCR alone cannot indicate protozoan viability. An alternative is to use messenger RNA (mRNA) for the PCR. The mRNA tends to have a short half life and therefore should not be present to be recovered from dead organisms (Wiedenmann *et al.*, 1998).

2.3 Alternative Indicator Organisms

2.3.1 Clostridium perfringens

Clostridium perfringens is a hardy, spore-forming bacterium that has potential use as an indicator of pathogenic bacteria, viruses, and protozoa. In wastewater treatment and disinfection evaluations, *C. perfringens* was found to be more disinfection-resistant than fecal coliform and *enterococcus*, and was a good indicator of the inactivation of *Cryptosporidium parvum* oocysts (Sobsey *et al.*, 1998b). It was also found to be a good indicator for human enteric viruses, *Cryptosporidium,* and *Giardia* in treated drinking water and river water (Payment and Franco, 1993). Research by Kueh *et al.* (1995) demonstrated correlations between gastrointestinal symptoms and concentrations of *Clostridium perfringens*. In marine waters it has been found to correlate with *Salmonella spp*. (Morinigo *et al.*, 1992) and *Giardia* and *Aeromonas* densities

(Ferguson *et al.*, 1996). *C. perfringens* has several desirable characteristics, including its presence in human feces but not bird droppings, and the superiority of spore survival to human pathogen survival. Furthermore, it can be easily and reliably enumerated using a membrane filter method.

2.3.2 Bacteriophages

Bacteriophages, viruses that infect bacteria, show promise as water quality indicators. Almost all bacteria known today have one or a group of specific bacteriophages that infect them. Coliphages are bacteriophages specific to coliform bacteria. As with *C. perfringens,* coliphages were found to be more resistant to disinfection than *E. coli,* fecal coliform and *enterococcus* in evaluations of wastewater treatment and chlorine disinfection (Farrah *et al.*, 1993; Sobsey *et al.*, 1998b).

Bacteriophages that infect through the bacterium's pili are called F+ (male-specific) phages, and bacteriophages that infect through the bacterium's membrane are called somatic phages. Studies have found F+ bacteriophages to be effective indicators of enteric virus concentrations in fresh waters (Havelaar *et al.*, 1993; Nasser and Oman, 1999). Lucena *et al.* (1996) suggested using phages of Bacteriodes fragilis, *C. perfringens,* and sometimes enteroviruses as indicators of persistent fecal pollution in marine sediments. In an urban estuarine study, however, F+ RNA bacteriophages did not correlate well with the pathogens measured (Ferguson *et al.*, 1996). Serrano *et al.* (1998) found that F+ RNA phages had low correlations with microbiological parameters in coastal waters, but that coliphages had statistically significant correlations with microbiological parameters. More evaluations are needed before a consensus will be reached regarding the selection and use of bacteriophages as indicators in various types of receiving waters.

2.4 Microbial Source Tracking

Attempts to reduce loads and prevent outbreaks via watershed management can be aided by accurate determination of the sources of microbial contamination. Microbial source tracking (MST) techniques can help give an indication of whether the sources of indicators or pathogens are human, wildlife, or agricultural. Categories of MST techniques include, among others, phenotypic and genetic methods, and may or may not require the development of a library of known samples for comparison with unknown samples. Drawbacks for MST methods include uncertainty in the spatial and temporal stabilities and variabilities of target characteristics. Ease of use and costs are also important in determining whether a method can be widely applied. While a summary is provided here, a critical review conducted by fellow EPA researchers (Simpson *et al.*, 2002) can be reviewed for more detailed information.

2.4.1 Antibiotic Resistance Analysis

Antibiotic resistance analysis (ARA) is a phenotypic method that takes advantage of the exposure of bacterial sources to different antibiotics and the resulting patterns of resistance that

develop. To determine a multiple antibiotic resistance (MAR) profile, a bacterial isolate is exposed to a suite of antibiotics. The antibiotics to which the isolate is resistant define the MAR profile, which acts as a fingerprint. First, a database of MAR profiles is acquired for samples of known sources in a given region. MAR profiles of unknown samples can then be compared to the database to determine their probable sources.

Wiggins (1996) analyzed 1,435 fecal streptococci isolates from animal and human sources for their resistance to five antibiotics. He then used discriminant analysis of the resulting patterns to classify the known isolates with a high rate of correct classification (92% of human isolates). Parveen *et al.* (1997) used MAR profiles to investigate *E. coli* sources within Apalachicola Bay and were able to identify MAR profile differences between point and nonpoint sources. Hagedorn *et al.* (1999) used antibiotic resistance in fecal streptococci to identify sources of nonpoint fecal pollution. Antibiotic resistance patterns have also been used in subtropical surface waters (Harwood *et al.*, 2000) and industrially perturbed stream waters (McArthur and Tuckfield, 2000). The analytical techniques for obtaining an antibiotic resistance profile are easy to perform. Antibiotic resistance patterns are, however, region-specific and compiling a MAR database of known sources is labor intensive. Furthermore, the MAR profiles of bacterial populations may shift with time. This approach may be best used in small watersheds with demonstrated nonpoint source problems and a limited number of potential sources (Simpson *et al.*, 2002).

2.4.2 Molecular Methods

The advance of molecular-based methods in recent years has aided source identification through the use of genetic markers. More commonly applied to microbial indicators because of their prevalence in the environment, these molecular-based MST methods are an active area of research and development. The review prepared by Simpson *et al.* (2002) describes the state of development of a number of techniques as well as their advantages and drawbacks. The genetic methods described in the review include ribotyping, length heterogeneity-PCR (LH-PCR), repetitive PCR (REP-PCR), denaturing gradient gel electrophoresis (DGGE), pulsed-field gel electrophoresis (PFGE), and amplified fragment length polymorphism (FLP). Although not yet ready for routine use, genetic methods are being tested in research studies. For example, a library-dependent PFGE was used to identify coliform sources in Northern Virginia's Four Mile Run Watershed (Simmons *et al.*, 2000). The study concluded that nonhuman species (waterfowl, raccoon, dog, deer, and Norway rat) were the primary *E. coli* sources in the urban stream. Human sources contributed only 18% of the *E. coli* (NVRC, 2002).

Because of the lack of a therapeutic cure or drug therapy for cryptosporidiosis, MST techniques for *Crytosporidium parvum* oocysts are particularly appealing. The Centers for Disease Control (CDC) has evaluated a molecular species- and strain-specific method for analyzing *Cryptosporidium* parasites in environmental samples (Royer *et al.*, 2002; Xiao *et al.*, 2000; Xiao *et al*., 2001). The method is a nested PCR-restriction fragment length polymorphism technique. It produces numerous copies of a targeted DNA sequence, uses an enzyme to break it into fragments and uses gel electrophoresis and staining to separate and visualize the fragments. Numerous *Cryptosporidium* species have been examined using this method. It has been tested

on stream water, surface water, and wastewater, and is claimed to be able to differentiate between potential sources such as humans, cattle, pets, and wildlife.

In storm stream flow in a mostly undeveloped and forested portion of the New York City watershed, the procedure identified no genotypes from humans or farm animals, indicating the genotypes were likely from wildlife. In raw surface water collected less than a mile downstream of a large commercial cattle operation and a wastewater treatment plant, the method confirmed the presence of *C. parvum* human and bovine genotypes. In Milwaukee, wastewater containing pretreated effluent from a large cattle slaughterhouse was found to contain several genotypes that were known to be associated with humans, bovines, dogs, cattle, and rodents. The method used by CDC to identify *Cryptosporidium* sources shows promise, but needs further development technologically and is as yet too expensive for routine monitoring (Xiao *et al.*, 2002; Royer *et al.*, 2002).

2.5 Conclusions

Speed, reasonable cost, accuracy, and the level of difficulty in performing the techniques remain considerations in the selection and execution of microbiological analyses for water quality. For analysis of total coliform, fecal coliform, *enterococcus*, and *E. coli*, membrane filtration methods are well established and straightforward to perform without specialized equipment. Disadvantages include length of analysis times and potential underestimation. Rapid commercial enzyme-based methods such as Colilert® and Enterolert® show promise for easy screening. This is especially useful in situations where water quality can change rapidly, requiring frequent testing. Users should initially test rapid methods against the traditional membrane filtration or most probable number techniques in order to check their technique and understand any limitations of the methods. Because *E. coli* O157:H7 lacks the enzyme β-glucuronidase, a separate test, such as an immunological method, is needed if its presence is suspected. Commercial gene probe kits are available for some bacteria such as *E. coli* and *Salmonella*. Commercial ELISA kits can also be purchased. These have been developed for food and clinical applications; their use for environmental samples can be explored.

Immunofluorescence and ELISA methods are currently available options for detection of nonculturable viruses and bacteria as well as *Cryptosporidium*, *Giardia*, and *E. coli* O157:H7. Commercially prepared ELISA kits are available for some viruses. Although not as sensitive as PCR-based techniques, immunological methods permit quantification. Allen *et al.* (2000) have warned, however, of limitations of the IF methods for *Cryptosporidium* and *Giardia*, including poor recoveries and inability to determine viability. Poor recoveries are an issue for viruses as well because elution efficiencies from filters can be low. Recovery may be less of an issue in the detection of bacteria, especially indicator bacteria, because they do not need to be retained and eluted from a filter for concentration. However, recovery and enumeration of pathogenic bacteria remains an issue when concentrations are low and exposure is high.

Problems with low viral and protozoan concentrations are being overcome by the high sensitivities of nucleic acid techniques, which include gene probes for detection and PCR for amplification of small amounts of a pathogen's DNA or RNA. The large number of research

studies using PCR in the detection of pathogens illustrates the versatility and promise of these methods. In particular, the ability to detect low concentrations is beneficial because of the low infectious doses of protozoa and viruses. PCR also permits detection of nonculturable viruses and viable but nonculturable bacteria. These methods are still at the research stage and they are not widely available, although they may be in the future. A major drawback to PCR-based methods is the inability to indicate viability; results should be considered evidence of recent contamination and should not necessarily imply risk. Expensive and specialized analytical needs are another drawback.

Although the ability to detect low concentrations of pathogens offers advantages in pathogen monitoring, results must be interpreted with care. The calculation of pathogen density from the analysis of a water sample is based on the assumption that the pathogens are distributed evenly in the water body being sampled. If this assumption is not true, then the absence of microorganisms in a sample may not mean that the organism is absent in the water. On the other hand, detection of a pathogen may give rise to an erroneously high estimate of pathogen density (Allen *et al.*, 2000). Furthermore, pathogen contamination may be transient and easily missed. Ongoing background sampling is important for establishing the normal microbiological conditions of a watershed; sampling should also be conducted when a disturbance such as a storm increases the likelihood of pathogen presence.

Detection methods are continually evolving, but direct routine monitoring for pathogens is not feasible at this time. Indicator use is far from ideal, but it still represents the most viable option for a basic level of water quality monitoring. Unfortunately, indicator bacteria make poor proxies for viruses and protozoa because their survival characteristics are different from those of viruses and protozoa. Potential incorporation of *C. perfringens* and bacteriophages into monitoring strategies may improve the representativeness of the indicator organisms. Because organisms such as *Aeromonas*, an opportunistic pathogen, and some fecal coliform have nonhuman sources, looking only for human-based fecal contamination does not cover all risk factors. MST techniques can allow watershed managers to determine whether the sources of indicator or pathogens are human, wildlife, or from domesticated animals. ARA is currently the easiest to execute, but in time genetic methods may play an increasing role in tracking down the microbiological sources of water quality impairments.

References

Abbott, S., B. Caughley, and G. Scott. (1998). Evaluation of Enterolert Registered for the Enumeration of *enterococci* in the Marine Environment. *New Zealand Journal of Marine and Freshwater Research* 32(4):505-513.

Allen, M.J., J.L. Clancy, and E.W. Rice. (2000). The Plain, Hard Truth About Pathogen Monitoring. *Journal of the American Water Works Association* 92(9):64-76.

Alvarez, A.J., E.A. Hernandez-Delgado, and Toranzos, G.A. (1993). Advantages and Disadvantages of Traditional and Molecular Techniques Applied to the Detection of Pathogens in Waters. *Water Science and Technology* 27(3-4):253-256.

Bej, A.K., R.J. Steffan, J. DiCesare, L. Haff, and R.M. Atlas. (1990). Detection of Coliform Bacteria in Water by Polymerase Chain Reaction and Gene Probes. *Applied and Environmental Microbiology* 56(2):307-314.

Bitton, G. (1980). *Introduction to Environmental Virology*. John Wiley and Sons, New York.

Budnick, G.E., R.T. Howard, and D.R. Mayo. (1996). Evaluation of Enterolert for Enumeration of *Enterococci* in Recreational Waters. *Applied and Environmental Microbiology* 62(10): 3881-3884.

Champliaud, D., P. Gobet, M. Naciri, O. Vagner, J. Lopez, J.C. Buisson, I. Varga, G. Harly, R. Mancassola, and A. Bonnin. (1998). Failure to Differentiate *Cryptosporidium Parvum* from *C. Meleagridis* Based on PCR Amplification of Eight DNA Sequences. *Applied and Environmental Microbiology* 64(4):1454.

Clesceri, L.S., A.E. Greenberg, and A.D. Eaton (Editors). (1998). *Standard Methods for the Examination of Water and Wastewater*, 20th Edition. American Public Health Association, American Water Works Association, and Water Environment Federation, Washington, DC.

DeLeon, R. and M.D. Sobsey. (1991). Methods for virus detection in water. In *Monitoring Water in the 1990s: Meeting New Challenges*, ASTM STP 1102, Editors: Hall, J.R. and D.G. Glysson. American Society for Testing and Materials, Philadelphia, PA.

Eckner, J.F. (1998). Comparison of Membrane Filtration and Multiple Tube Fermentation by the Colilert and Enterolert Methods for Detection of Waterborne Coliform Bacteria, *Escherichia Coli*, and *Enterococci* Used in Drinking and Bathing Water Quality Monitoring in Southern Sweden. *Applied and Environmental Microbiology* 64(8):3079-3083.

Edberg, S.C., M.J. Allen, and D.B. Smith. (1989). Rapid, Specific Autoanalytical Method for the Simultaneous Detection of Total Coliforms and *E. Coli* from Drinking Water. *Water Science and Technology* 21(3):173-177.

Edberg, S.C., M.J. Allen, D.B. Smith, and N.J. Kriz. (1990). Enumeration of Total Coliforms and *Escherichia Coli* from Source Water by the Defined Substrate Technology. *Applied and Environmental Microbiology* 56(2):366-369.

Farrah, S.R., S.H. Cleaver, and P.J. London. (1993). Removal of Microorganisms by an Advanced Wastewater Reclamation Facility. General Meeting of the *American Society for Microbiology* 93(0):426.

Ferguson, C.M., B.G. Coote, N.J. Ashbolt, and. I.M. Stevenson. (1996). Relationships Between Indicators, Pathogens and Water Quality in an Estuarine System. *Water Research* 30(9): 2045-2054.

Francy, D.S. and R.A. Darner. (2000). Comparison of Methods for Determining *Escherichia coli* Concentrations in Recreational Waters. *Water Research* 34(10): 2770-2778.

Fricker, E.J., K.S. Illingworth, and C.R. Fricker. (1997). Use of Two Formulations of Colilert and Quantitray for Assessment of the Bacteriological Quality of Water. *Water Research* 31(10): 2495-2499.

Fricker, E.J., K. Murrin, and C.R. Fricker. (1999). Use of PCR for Detection of Bacteria and Viruses, *Water Supply* 17(2):5.

Gantzer, C., S. Senouci, A. Maul, Y. Levi, and L. Schwartzbrod. (1999). Enterovirus Detection from Wastewater by RT-PCR and Cell Culture. *Water Science and Technology* 40 (2):105.

Genthe, B., M. Gericke, B. Bateman, N. Mjoli, and R. Kfir. (1995). Detection of Enteric Adenoviruses in South African Waters Using Gene Probes. *Water Science and Technology* 31(5-6):345.

Gerba, C.P., A.B. Margolin, and M.J. Hewlett. (1989). Application of Gene Probes to Virus Detection in water. *Water Science and Technology* 21(3):147.

Gilgen, M., B. Wegmueller, P. Burkhalter, H-P. Buehler, U. Mueller, J. Luethy, and U. Candrian. (1995). Reverse Transcription PCR to detect enteroviruses in surface water. *Applied and Environmental Microbiology* 61(4):1226.

Greening, G.E., L. Woodfield, and G.D. Lewis. (1999). RT-PCR and Chemiluminescent ELISA for Detection of Enteroviruses. *Journal of Virological Methods* 82:157-166.

Griffin, D.W., C.J. Gibson III., E.K. Lipp, K. Riley, J.H. Paul III., and J.B. Rose. (1999). Detection of Viral Pathogens by Reverse-Transcriptase PCR and of Microbial Indicators by Standard Methods in the Canals of the Florida Keys. *Applied and Environmental Microbiology* 65(9): 4118-4125.

Hagedorn, C., S.L. Robinson, J.R. Filtz, S.M. Grubbs, T.A. Angier, and R.B. Reneau, Jr. (1999). Determining Sources of Fecal Pollution in a Rural Virginia Watershed with Antibiotic Resistance Patterns in Fecal *Streptococci*. *Applied and Environmental Microbiology* 65 (12):5522-5531.

Hallier-Soulier, S. and E. Guillot. (1999). An Immunomagnetic Separation Polymerase Chain Reaction Assay for Rapid and Ultra Sensitive Detection of *Cryptosporidium Parvum* in Drinking Water. *FEMS Microbiology Letters* 176(2):285.

Harwood, V.J., J. Whitlock, and V. Withington. (2000). Classification of Antibiotic Resistance Patterns of Indicator Bacteria by Discriminant Analysis: Use in Predicting the Source of Fecal Contamination in Subtropical Waters. *Applied and Environmental Microbiology* 66 (9):3698-3704.

Havelaar, A.H., M. Van Olphan, and Y.C. Drost. (1993). F-Specific RNA Bacteriophages are Adequate Model Organisms for Enteric Viruses in Fresh Water. *Applied and Environmental Microbiology* 59(9):2956-2962.

Huq, A. and. R. Colwell. (1996). A Microbiological Paradox: Viable but Nonculturable Bacteria with Special Reference to *Vibrio Cholerae*. *Journal of Food Protection* 59(1):96-101.

Hurst, C.J., W.H. Benton, and R.E. Stetler. (1989). Detecting Viruses in Water. *Journal of the American Water Works Association* 81 (9):71.

Ivnitski, D., I. Abdel-Hamid, P. Atanasov, and E. Wilkins. (1999). Biosensors for Detection of Pathogenic Bacteria. *Biosensors and Bioeletronics* 14(7):559.

Jarmey-Swan, C., R.A. Gibbs, G.E. Ho, I.W. Bailey, and A.R. Howgrave-Graham. (2000). A Novel Method for Detection of vVable *Giardia* Cysts in Water Samples. *Water Research* 34(6):1948.

Kfir, R., M. du Preez, and B. Genthe. (1993). The Use of Monoclonal Antibodies for the Detection of Fecal Bacteria in Water. *Water Science and Technology* 27(3-4):257-260

Kfir, R. and B. Genthe. (1993). Advantages and Disadvantages of the Use of Immunodetection Techniques for the Enumeration of Microorganisms and Toxins in Water. *Water Science and Technology* 27(3-4), 243.

Kong, R.Y.C., A. Pelling, C.L. So, and R.S.S. Wu. (1999a). Identification of Oligonucleotide Primers Targeted at the 16S-23S rDNA Intergenic Spacers for Genus- and Species-Specific Detection of Aeromonads, *Marine Pollution Bulletin* 38(9):802.

Kong, R.Y.C., C.L. So, W.F. Law, and R.S.S. Wu. (1999b). A Sensitive and Versatile Multiplex PCR System for the Rapid Detection of Enterotoxigenic (ETEC), Enterohaemorrhagic (EHEC), and Enteropathogenic Strains of *Escherichia coli*. *Marine Pollution Bulletin* 38 (12):1207.

Kopecka, H., S. Dubrou, J. Prevot, J. Marechal, and J.M. Lopez-Pila. (1993). Detection of Naturally Occurring Enteroviruses in Waters by Reverse Transcription, Polymerase Chain Reaction, and Hybridization. *Applied and Environmental Microbiology* 59(4):1213-1219.

Kozwich, D., K.A. Johansen, K. Landau, C.A. Roehl, S. Wononoff, and P.A. Roehl. (2000). Development of a Novel, Rapid Integrated *Cryptosporidium Parvum* Detection Assay. *Applied and Environmental Microbiology*. 66(7): 2711-2717.

Kueh, C.S.W., T-Y. Tam, T.W. Lee, S.L. Wang, O.L. Lloyd, I.T.S. Yu, T.W. Wang, J.S. Tam, and D.C.J. Bassett. (1995). Epidemiological Study of Swimming-Associated Illnesses Relating to Bathing-Beach Water Quality. *Water Science and Technology* 31:1-4.

Lucena, F., R. Araujo, and J. Jofre. (1996). Usefulness of Bacteriophages Infecting Bacteriodes Fragilis as Index Microorganisms of Remote Faecal Pollution. *Water Research* 30 (11): 2812-2816.

Margolin, A.B., M. J. Hewlett, and C.P. Gerba. (1990). The Application of a Poliovirus cDNA Probe for the Detection of Enteroviruses in Water. *Water Science and Technology* 24(2): 277.

Margolin, A.B., C.P. Gerba, K.J. Richardson, and J. E. Naranjo. (1993). Comparison of Cell Culture and a Poliovirus Gene Probe Assay for the Detection of Enteroviruses in Environmental Water Samples. *Water Science and Technology* 27(3-4):311.

Marx, F.E., M.B. Taylor, and W.O.K. Grabow. (1998). The Application of a Reverse Transcriptase-Polymerase Chain Reaction-Oligonucleotide Probe Assay for the Detection of Human Astroviruses in Environmental Water. *Water Research* 32(7):2147.

McArthur, J.V. and R.C. Tuckfield. (2000). Spatial Patterns in Antibiotic Resistance Among Stream Bacteria: Effects of Industrial Pollution. *Applied and Environmental Microbiology* 66(9):3722-3726.

McFeters, G.A., B.H. Pyle, J.T. Lisle, and S.C. Broadway. (1999). Rapid, direct methods for enumeration of specific, active bacteria in water and biofilms. *Journal of Applied Microbiology Symposium Supplement* 85:193S.

Meckes, M. (2001). Personal communication. U.S. EPA, 26 West Martin Luther King Drive, Cincinnati, OH.

Metcalf, T.G., J.L. Melnick, and M.K. Estes. (1995). Environmental Virology: From Detection of Virus in Sewage and Water by Isolation to Identification by Molecular Biology: A trip of Over 50 Years. *Annual Reviews in Microbiology* 49: 461-487.

Morinigo, M.A., M.A. Munoz, R. Cornax, E. Martinez-Manzanares, and J.J. Borrego. (1992). Presence of Indicators and Salmonella in Natural Waters Affected by Outfall Wastewater Discharges. *Water Science and Technology* 25(9):1-8.

Nasser, A.M., Y. Elkana, and L. Goldstein. (1993). A Nylon Filter A-ELISA for Detecting Viruses in Water. *Water Science and Technology* 27(7-8):135.

Nasser, A.M., M.K. Estes, and T.G. Metcalf. (1991). Detection of Human Rotaviruses in Fresh and Estuarine Waters by Dot-Blot Hybridization. *Water Science and Technology* 23(1-3):253.

Nasser, A.M. and T.G. Metcalf. (1987). An A-ELISA to Detect Hepatitis A virus in Estuarine Samples. *Applied and Environmental Microbiology* 53(5):1192.

Nasser, A.M. and S.D. Oman. (1999). Quantitative Assessment of the Inactivation of Pathogenic and Indicator Viruses in Natural Water Sources. *Water Research* 33(7):1748-1752.

Nasser, A.M., Y. Tchorch, and B. Fattal. (1994). Validity of Serological Methods (ELISA) for Detecting Infectious Viruses in Water. *Water Science and Technology* 31(5-6):307.

Northern Virginia Regional Commission. (2002). *Fecal Coliform TMDL (Total Maximum Daily Load) Development for Four Mile Run, Virginia*, www.novaregion.org/4MileRun/TMDL/4mr_TMDL_5-31-02.pdf. Prepared for: Virginia Department of Environmental Quality and Virginia Department of Conservation and Recreation, First Submission: March 21, 2002; Revised: April 25, 2002; Accepted May 31, 2002.

Oragui, J.I, D.D. Mara, S.A. Silva, and A.M. Konig. (1989). New technique for the Enumeration of Rotaviruses in Wastewater Samples. *Water Science and Technology* 21(3):99.

Palmer, C.J., Y-L. Tsai, C. Paszko-Kolova, C. Mayer, and L.R. Sangermano. (1993). Detection of Legionella Species in Sewage and Ocean Water by Polymerase Chain Reaction, Direct Fluorescent-Antibody, and Plate Culture Methods. *Applied and Environmental Microbiology* 59(11):3618-3624.

Parveen, S., R.L. Murphree, L. Edmiston, C.W. Kaspar, K.M. Portier, and M.L. Tamplin. (1997). Association of Multiple-Antibiotic-Resistance Profiles with Point and Nonpoint Sources

of *Escherichia Coli* in Apalachicola Bay. *Applied and Environmental Microbiology* 63(7):2607-2612.

Payment, P. and E. Franco. (1993). *Clostridium perfringens* and Somatic Coliphages as Indicators of the Efficiency of Drinking Water Treatment for Viruses and Protozoan Cysts. *Applied and Environmental Microbiology* 59(8):2418-2424.

Prescott, A.M., A. Jonas, and C.R. Fricker. (1999). In Situ Hybridization Studies for the Detection of Micro-Organisms in the Environment. *Water Supply* 17(2):17.

Pyle, B.H., S.C. Broadaway, and G.A. McFeters. (1995). A Rapid, Direct Method for Enumerating Respiring Enterohemorrhagic *Escherichia coli* O157:H7 in Water. *Applied and Environmental Microbiology* 61(7):2614-2619.

Pyle, B.H., S.C. Broadaway, and G.A. McFeters. (1999). Sensitive Detection of *Escherichia Coli* O157:H7 in Food and Water by Immunomagnetic Separation and Solid-Phase Laser Cytometry. *Applied and Environmental Microbiology* 65(5).

Rice, E.W., T.C. Covert, S.A. Johnson, C.H. Johnson, and D.L. Reasoner. (1995). Detection of *Escherichia Coli* in Water Using a Colorimetric Gene Probe Assay. *Journal of Environmental Science and Health* A30(5):1059.

Robertson, W., G. Palmateer, J. Aldom, and D.Van Bakel. (1998). Evaluation of a Rapid Method for *E. Coli* and Thermotolerant Coliforms in Recreational Waters. *Water Science and Technology* 38(12):87-90.

Rochelle, P.A., R. Deleon, M.H. Stewart, and R.L. Wolfe. (1997). In Situ PCR for Detection of Infectious *Cryptosporidium Parvum* in Water. *American Society of Microbiology*, General Meeting Abstracts (Q22):459.

Rose, J.B. (1997). Environmental Ecology of *Cryptosporidium* and Public Health Implications. *Annual Review Public Health*.18:135-161.

Royer, M., L. Xiao and A. Lal. (2002). Animal Source Identification Using a *Cryptosporidium* DNA Characterization Technique, EPA/600/R-03/047. U.S. EPA Office of Research and Development, Cincinnati, OH.

Sartory, D.P. and J. Watkins. (1999). Conventional Culture for Water Quality Assessment: is there a Future? *Journal of Applied Microbiology Symposium Supplement* 85:225S-233S.

Schnattinger, A. (1985). Detection of Hepatitis A Virus in Drinking Water by Enzyme-Immunoassay Using Ultracentrifugation for Virus Concentration. *Water Science and Technology* 17(10): 39.

Schwab, D.J., R. De Leon, and M.D. Sobsey. (1993). Development of PCR Methods for Enteric Virus Detection in Water. *Water Science and Technology* 27(3-4):211-218.

Schwab, K.J., R. DeLeon, and M.D. Sobsey. (1996). Immunoaffinity Concentration and Purification of Waterborne Enteric Viruses for Detection by Reverse Transcriptase PCR. *Applied and Environmental Microbiology* 62(6):2086-2094.

Serrano, E., B. Moreno, M. Solaun, J.J. Aurrekoetxea, and J. Ibarluzea. (1998). The Influence of Environmental Factors on Microbiological Indicators of Coastal Water Pollution. *Water Science and Technology* 38(12):195-199.

Shadix, L.C. and E.W. Rice. (1991). Evaluation of β-Glucuronidase Assay for the Detection of *Escherichia Coli* from Environmental Waters. *Canadian Journal of Microbiology* 37: 908-911.

Shieh, Y.-S. C., R. Baric, M.D. Sobsey, J. Ticehurst, T.A. Miele, R. DeLeon, and R. Walter. (1991). Detection of Hepatitis A Virus and other Enteroviruses by ssRNA Probes. *Journal of Virological Methods* 31:119-136.

Simmons, G. M., D. F. Waye, S. Herbein, S. Myers, and E. Walker. (2000). Estimating Nonpoint Fecal Coliform Sources in Northern Virginia's Four Mile Run Watershed. In T. Younos and J. Poff (ed.) Abstracts, *Virginia Water Research Symposium 2000 VWRRC Special Report*, SR-19-2000. 248-267. Blackburg, VA.

Simpson, J.M., J.W. Santo Domingo, D.J. Reasoner. (2002). Microbial Source Tracking: State of Science. *Environmental Science and Technology* 36(24):5279-5288.

Slifko, T.R., D. Friedman, J.B. Rose, and W. Jakubowski. (1997). An In Vitro Method for Detecting Infectious *Cryptosporidium* Oocysts with Cell Culture. *Applied and Environmental Microbiology* 63(9):3669-3675.

Slifko, T.R., D.E. Huffman, and J.B. Rose. (1999). A Most-Probable-Number Assay for Enumeration of Infectious *Cryptosporidium Parvum* Oocysts. *Applied and Environmental Microbiology* 65(9):3936-3941.

Sobsey, M.D., D.A. Battigelli, G-A. Shin, and S. Newland. (1998a). RT-PCR Amplification Detects Inactivated Viruses in Water and Wastewater. *Water Science and Technology* 38 (12): 91.

Sobsey, M.D., M.J. Casteel, H. Chung, G. Lovelace, O.D. Simmons III, F. Hsu, and J.S.Meschke. (1998b). Innovative Technologies for Waste Water Disinfection and Pathogen Detection. In Proceedings of: *Disinfection 1998 -The Latest Trends in Wastewater Disinfection: Chlorination vs. UV Disinfection*, Baltimore, MD.

Soule, H., O. Genoulaz, B. Gratacap-Cavallier, P. Chevallier, J-X. Liu, and J-M. Seigneurin. (2000). Ultrafiltration and Reverse Transcription-Polymerase Chain Reaction: an Efficient Process for Poliovirus, Rotavirus and Hepatitis A Virus Detection in Water. *Water Research* 34(3):1063.

Stinear, T., A. Matusan, K. Hines, and M. Sandery. (1996). Detection of a Single Viable *Cryptosporidium Parvum* Oocyst in Environmental Water Concentrates by Reverse transcription PCR. *Applied and Environmental Microbiology* 62(9):3385.

Townsend, D.E., and A.J. Crouteau, E.E. Ehrenfeld, and A. Naqui. (1996). Colilert-18™: A New Test Designed to Detect Total Coliforms and *Escherichia Coli* in Drinking Water After 18 Hours of Incubation. *American Society for Microbiology*, General Meeting Abstracts, Session 264:463.

Toze, S. (1999). PCR and the Detection of Microbial Pathogens in Water and Wastewater. *Water Research* 33(17):3545-3556.

Tsai, Y-L, B. Tran, L.R. Sangermano, and C.J. Palmer. (1994). Detection of Poliovirus, Hepatitis A Virus, and Rotavirus from Sewage and Ocean Water by Triplex Reverse Transcriptase PCR. *Applied and Environmental Microbiology* 60(7):2400-2407.

Tsen, H.Y., C.K. Lin, and W.R. Chi. (1998). Development and Use of 16S rRNA Gene Targeted PCR Primers for the Identification of *Escherichia Coli* Cells in Water. *Journal of Applied Microbiology* 85(3):554.

U.S. EPA. (1985). *Test Methods for Escherichia Coli and Enterococci in Water by the Membrane-Filter Procedure*, EPA-600/4-85-076. Office of Research and Development, Environmental Monitoring and Support Laboratory, Cincinnati, OH.

U.S. EPA. (1997). *Method 1600: Membrane Filter Test Method for Enterococci in Water*, EPA-821-R-97-004a. Office of Water, Washington, DC.

U.S. EPA. (1999). *Control of Pathogens and Vector Attraction in Sewage Sludge*, EPA/625/R-92/013. Office of Research and Development, Washington, DC.

U.S. EPA. (2001). *Method 1623: Cryptosporidium and Giardia in Water by Filtration/MS/FA*, EPA-821-R-01-025. Office of Water, Washington, DC.

Vantarakis, A.C. and M. Papapetropoulou. (1998). Detection of Enteroviruses and Adenoviruses in Coastal Waters of SW Greece by Nested Polymerase Chain Reaction. *Water Research* 32(8):2365-2372.

Vantarakis, A.C. and M. Papapetropoulou. (1999). Detection of enteroviruses, adenoviruses and Hepatitis A viruses in raw sewage and treated effluents by nested-PCR. *Water, Air, and Soil Pollution* 114(1-2):85-93.

Vesey, G., N. Ashbolt, E.J. Fricker, D. Deere, K.L. Williams, D.A.Veal, and M. Dorsch. (1998). The Use of a Ribosomal RNA Targeted Oligonucleotide Probe for Fluorescent Labeling of Viable *Cryptosporidium Parvum* Oocysts. *Journal of Applied Microbiology* 85(3):429.

Ware, M., M. Rodgers, P. Scarpino, C.T. Yamashiro, C. Paszko-Kolva, and W. Jakubowski. (1995). Development and Evaluation of a PCR Detection Method for *Giardia* and *Cryptosporidium* in Water Samples. *American Society for Microbiology*, General Meeting Abstracts, Session 151: 436.

Way, J.S., K.L. Josephson, S.D. Pillai, A. Abbaszadegan, C.P. Gerba, and I.L.Pepper. (1993). Specific Detection of *Salmonella Spp.* by Multiplex Polymerase Chain Reaction. *Applied and Environmental Microbiology* 59(5):1473-1479.

Wiedenmann, A., P. Kruger, and K. Botzenhart. (1998). PCR Detection of *Cryptosporidium parvum* in environmental samples - a review of published protocols and current developments. *Journal of Industrial Microbiology and Biotechnology* 21, 150-166.

Wiggins, B.A. (1996). Discriminant Analysis of Antibiotic Resistance Patterns in Fecal Streptococci, a Method to Differentiate Human and Animal Sources of Fecal Pollution in Natural Waters. *Applied and Environmental Microbiology* 62(11):3997-4002.

Xiao, L., K. Alderisio, J. Limor, M. Royer, and A.A. Lal. (2000). Identification of Species and Sources of *Cryptosporidium* Oocysts in Stormwaters with a Small- Subunit rRNA-Based Diagnostic and Genotyping Tool. *Appl. Environ. Microbiol*. 66(12): 5492-5498.

Xiao, L., A. Singh, J. Limor, T. Graczyk, S. Gradus, and A.A. Lal. (2001). Molecular Characterization of *Cryptosporidium* Oocysts in Samples of Raw Surface Water and Wastewater. *Appl. Env. Microbiol* 67:1097-1101.

Xiao, L., M. Royer, and A.A. Lal. (2002). Molecular Detection of *Cryptosporidium* Oocysts in Water: the Challenge and Promise. Abstract for WQTC Conference, November 2002.

Yu, H. and J.G. Bruno. (1996). Immunomagnetic-Electrochemiluminescent Detection of *Escherichia Coli* O157 and *Salmonella Typhimurium* in Foods and Environmental Water Samples. *Applied and Environmental Microbiology* 62(2):587-592.